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UTILITY PATENT APPLICATION TRANSMITTAL

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Washington, D.C. 20231

Attorney Docket No. 205970

First Named Inventor Arnd BAUMANN

Express Mail No. EL643535335US

APPLICATION ELEMENTS	ACCOMPANYING APPLICATION PARTS
1. ☒ Utility Transmittal Form	8. ☐ Assignment Papers (cover sheet and document(s))
2. ☒ Specification (including claims and abstract) [Total Pages 47]	9. ☐ Power of Attorney
3. ☒ Drawings [Total Sheets 18]	10. ☐ English Translation Document (if applicable)
4. ☐ Combined Declaration and Power of Attorney [Total Pages]	11. ☐ Information Disclosure Statement (IDS) ☐ Form PTO-1449 ☐ Copies of References
a. ☐ Newly executed	12. ☐ Preliminary Amendment
b. ☐ Copy from prior application **[Note Box 5 below]**	13. ☒ Return Receipt Postcard (Should be specifically itemized)
i. ☐ Deletion of Inventor(s) Signed statement attached deleting inventor(s) named in the prior application	14. ☐ Small Entity Statement(s) ☐ Enclosed ☐ Statement filed in prior application; status still proper and desired
5. ☐ Incorporation by Reference: The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	15. ☐ Certified Copy of Priority Document(s)
6. ☐ Microfiche Computer Program	16. ☒ Other: Patent Application Cover Sheet
7. ☒ Nucleotide and/or Amino Acid Sequence Submission	
a. ☐ Computer Readable Copy	
b. ☒ Paper Copy	
c. ☐ Statement verifying above copies	

17. **If a CONTINUING APPLICATION**, check appropriate box and supply the requisite information in (a) and (b) below:

(a) ☐ Continuation ☐ Divisional ☒ Continuation-in-part of prior application Serial No. PCT/EP99/00942 filed February 12, 1999.
Prior application information: Examiner ; Group Art Unit:

(b) Preliminary Amendment: Relate Back - 35 USC §120. The Commissioner is requested to amend the specification by inserting the following sentence before the first line:
"This is a ☒ continuation-in-part ☐ divisional of copending application(s)
☐ Application No. , filed on .
☒ International Application No. PCT/EP99/00942, filed on February 12, 1999, and which designates the U.S."

APPLICATION FEES

BASIC FEE					$690.00
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total Claims	46	-20=	26	x $18.00	$468.00
Independent Claims	5	- 3=	2	x $78.00	$156.00
☐ Multiple Dependent Claims(s) if applicable				+$260.00	$
			Total of above calculations =		$1,314.00
			Reduction by 50% for filing by small entity =		$(0.00)
☐ Assignment fee if applicable				+ $40.00	$
				TOTAL =	$1,314.00

UTILITY PATENT APPLICATION TRANSMITTAL | Attorney Docket No. 205970

19. ☒ Please charge my Deposit Account No. 12-1216 in the amount of $1,314.00.

20. ☐ A check in the amount of $ is enclosed.

21. The Commissioner is hereby authorized to credit overpayments or charge any additional fees of the following types to Deposit Account No. 12-1216:
    a. ☒ Fees required under 37 CFR §1.16.
    b. ☒ Fees required under 37 CFR §1.17.

22. ☒ The Commissioner is hereby generally authorized under 37 CFR §1.136(a)(3) to treat any future reply in this or any related application filed pursuant to 37 CFR §1.53 requiring an extension of time as incorporating a request therefor, and the Commissioner is hereby specifically authorized to charge Deposit Account No. 12-1216 for any fee that may be due in connection with such a request for an extension of time.

23. CORRESPONDENCE ADDRESS

☒ Customer Number: 23460 23460 PATENT TRADEMARK OFFICE	☐ , Reg. No. Leydig, Voit & Mayer, Ltd. Two Prudential Plaza, Suite 4900 180 North Stetson Chicago, Illinois 60601-6780 (312) 616-5600 (telephone) (312) 616-5700 (facsimile)

Name	Carol Larcher, Registration No. 35,243
Signature	Carol Larcher
Date	August 17, 2000

Certificate of Mailing Under 37 CFR §1.10

I hereby certify that this Utility Patent Application Transmittal and all accompanying documents are being deposited with the United States Postal Service "Express Mail Post Office To Addressee" Service under 37 CFR §1.10 on the date indicated below and is addressed to: Commissioner of Patents and Trademarks, Box Patent Application, Washington, D.C. 20231.

Irina Mikitiouk	I. Mikitiouk	August 17, 2000
Name of Person Signing	Signature	Date

Attorney Docket No.
205970

PATENT APPLICATION

Invention Title:
SEQUENCES OF AN I_H ION CHANNEL AND USE THEREOF

Inventors:

INVENTOR'S NAME	CITIZENSHIP	CITY OF RESIDENCE	STATE or FOREIGN COUNTRY
Arnd BAUMANN	Germany	Juelich	Germany
Wolfgang BONIGK	Germany	Juelich	Germany
Renate GAUSS	Germany	Juelich	Germany
Alexander SCHOLTEN	Germany	Dormagen	Germany
Reinhard SEIFERT	Germany	Aachen	Germany
Benjamin KAUPP	Germany	Aachen	Germany

Be it known that the inventors listed above have invented a certain new and useful invention with the title shown above of which the following is a specification.

Sequences of an I_h ion channel and use thereof

The present invention relates to a nucleic acid, preferably a DNA, comprising at least part of the sequence of an I_h ion channel. Said sequence may e.g. be derived from a human DNA, a rat DNA, a bovine DNA, a *Drosophila melanogaster* DNA or a sea urchin DNA. Furthermore, the present invention relates to an mRNA molecule which contains the corresponding sequences. The invention further relates to a polypeptide or protein comprising the corresponding derived amino acid sequence.

Furthermore, the invention relates to the use of one or more of the above-mentioned sequences in a screening and/or diagnosing method and to the kits required therefor.

Lastly, the invention relates to the use of one or more of the above-mentioned sequences for the treatment and/or prophylaxis of cardiovascular disorders and sleep disturbances.

The many different functions of the nerve system are substantially determined by finely adjusted interactions between the intrinsic characteristics of the neurons and the synaptic connections. The electrophysiological characteristics inherent to the neurons and synapses are, in turn, determined by the localization and density of the voltage- and ligand-controlled ion channels which regulate the flow of ion currents across the neuronal plasma membrane and which are controlled by a great number of transmitter substances and intracellular messenger systems (Hille, 1992).

With regard to the specific activity expected of the neuronal elements, it is not astonishing that neurons have a great repertory of ion channels, including the classic

channels that produce voltage-dependent sodium (NA^+) and potassium (K^+) currents during an action potential (Hodgkin and Huxley, 1952) and also a number of unusual ion conductances (Linás, 1988).

An unusual intrinsic mechanism which had originally been discovered by Ito and colleagues (Araki et al., 1962; Ito and Oshima, 1965) in motoneurons of cats turned out to be a slow relaxation of the potential change induced by hyperpolarizing current, resulting in a non-ohmic behavior of the current/voltage (I/V) relationship in hyperpolarizing direction. The underlying time-dependent membrane current was first characterized in photoreceptors of the rods as cesium (Cs^+)-sensitive inward current which is triggered by hyperpolarization and may depolarize the membrane. This leads to the typical sequence of an initial transient hyperpolarization by exposure, followed by a slow depolarization (Attwell and Wilson, 1980; Bader et al., 1982; Bader et al., 1979; Fain et al., 1978).

The current in the photoreceptors was designated as I_h because it is activated by hyperpolarization. At about the same time a similar ion current was discovered in the heart, in the pacemaker cells of the sinus node and in the Purkinje fibers of the mammalian heart (Brown and DiFrancesco, 1980; Brown et al., 1979; DiFrancesco, 1981a; DiFrancesco, 1981b; Yanagihara and Irisawa, 1980), and it became clear that the slow inward current is accompanied by sodium and potassium ions. This current was called "funny" current (I_f) to emphasize its unusual behavior, i.e., the fact that an inward current is concerned which is activated by hyperpolarization and, oddly enough, was similar to the previously described K^+ conductance I_{K2}. There is a growing interest in said current because it participates, for instance, in the generation and control of spontaneous activity of the heart.

Further evidence of the presence of a corresponding current in central neurons was found, and it was mentioned by Halliwell and Adams (1982) for the first time. They observed a slow inward current, which was designated as "queer" current (I_q), in pyramidal cells of the hippocampus after hyperpolarization. Subsequently, currents with similar characteristics were found in a great number of neuronal and non-neuronal cells, and said hyperpolarization-activated current was finally recognized as an omnipresent phenomenon in cells of the nerve system. The designation as "I_h" is now accepted as a term for describing said current.

Although it was first assumed that the activity of the respective I_h channels is not modulated, more and more data show that the I_h channels are important targets for neurotransmitters and messenger systems, which emphasizes their important physiological role in the control of cellular electrical activities.

In the meantime it has become known that I_h significantly contributes to the rest potential, limits an excessive hyperpolarization, determines the form of action patterns (firing patterns) and takes part in the generation of rhythmic oscillations of the membrane potential. I_h currents have a few special characteristics that distinguish the same from other voltage-controlled ion channels. Like voltage-controlled Na^+, Ca^{2+} and specific K^+ currents, they have a steep voltage-dependence curve and activate with a sigmoidal time course; they are however activated by hyperpolarization and deactivate by sigmoidal kinetics.

The activation in negative potentials and the blockage by Cs^+ ions reminds of inwardly rectifying K^+ channels. However, many characteristics of I_h clearly differ from that K^+ channel family: The activation kinetics is slower, the activation range is more positive and is independent of the extracellular K^+ concentration, conductance is substantially resistant to extracellular Ba^{2+} ions and the I_h channels are permeable not only to K^+ ions,

but also to Na^+ ions. In contrast to other cation channels, such as ligand-controlled cation channels, the I_h channels are very selective for Na^+ and K^+ ions and have a steep voltage-dependent control.

Of particular importance to the present research work is the participation of the I_h channels in the pacemaker function in the cardiac muscle. The pacemaker activity in the heart is due to specialized myocytes that are located in specific regions of the heart (*sinus venosus*) and are characterized by their ability to beat spontaneously even if separated from the rest of the cardiac muscle. In pacemaker cells of the sinus node in mammals, the spontaneous activity follows from a typical phase of their action potential, the slow diastolic depolarization. During said phase, which corresponds to the diastole of the cardiac contraction cycle, the membrane depolarizes again at a slow pace after termination of the action potential until the threshold value for the generation of a new action potential is reached. Thus the diastolic depolarization is responsible for the initiation of the rhythmic behavior and characterizes action potentials of the sinus node and other spontaneously active cardiocytes.

Apart from the generation of a rhythmic activity, the diastolic (or pacemaker) depolarization takes part in the control of the heartbeat frequency by autonomous neurotransmitters. It is known that the stimulation of the sympathic and parasympathic nerve system leads to an acceleration and deceleration of the heartbeat.

It has become known in the meantime that the I_h channels take part in this pacemaker function. The I_h current of the sinus node is an unspecific cation current, normally accompanied by Na^+ and K^+, which after hyperpolarization slowly activates in a voltage range encompassing that of the diastolic depolarization. The I_h features are well suited for producing a depolarization process as a reaction to a hyperpolarization in a voltage range in which the I_h channel is activated.

So far, however, it has not been possible to identify sequences of genes coding for I_h ion channels. Furthermore, channel protein has so far not been available in a sufficient amount for characterizing the same biochemically. Finally, the pharmacological characterization of I_h channels has so far been extremely difficult because the I_h currents were identified on whole cells, which additionally exhibit K^+- and Na^+-selective conductivities, and were experimentally isolated from the other currents.

It has therefore been the object of the present invention to indicate the nucleic acid, to show its possible applications, and to provide the protein in a functional state and in a sufficient amount for biochemical analyses and pharmaceutical applications.

Said object is achieved by the subject matter of the independent claims. Advantageous developments are indicated in the dependent claims.

The terms used hereinafter shall have the following meanings:

"I_h ion channel" is here to stand for those ion channels that (1) open by hyperpolarization and are closed at more positive voltage values ($V_m \geq$ -10 mV); (2) whose activation and deactivation take place with a relatively slow sigmoidal time course; (3) conduct not only K^+ ions, but also Na^+ ions; (4) are almost entirely blocked by 0.1 – 3 mM extracellular Cs^+ and (5) are directly modulated by cyclic nucleotides, in particular cyclo AMP and cyclo GMP.

"Stringent conditions" means hybridization with 0.1-5 x SSC, preferably 1-2 x SSC, at 60-70°C, preferably 65°C.

"Conditions of low stringency" means hybridization at 0.1-5 x SSC, preferably 1-2 x SSC at 50-60°C, preferably at 55°C.

"Parts" of the I_h ion channel means a section of the protein sequence suited as antigenic determinant, for example, a section of at least 6 amino acids. Sections that occur in the form of domains, such as the sections S1, S2, etc. as indicated in Fig. 1A, are also regarded as parts. This encompasses sections of the ion channel that derive from the DNA sequences indicated in SEQ ID NO 1 to 15 using the IUPAC code, namely by way of amino acid exchanges, deletions and additions, while maintaining the biological function.

"Part" thereof in connection with the nucleic acid means a fragment having a length of at least 6 nucleotides, preferably 12 nucleotides, particularly preferably a length of 18 nucleotides. The part is suited for hybridizing via oligonucleotide hybridization specifically (selectively) with the corresponding total sequence. Thus a "part" of the nucleic acid is a section from the sequences according to SEQ ID NO 1 to 15 that is suited for selectively hybridizing with one of the said sequences.

"Selectively" (specifically) means that under suitable hybridization conditions a nucleic acid only hybridizes with one nucleic acid as is indicated by one of the sequences according to SEQ ID NO 1 to 15, whereas it does not hybridize with another nucleic acid of the respective host organism with which it is normally associated.

"Homology" as is here used is calculated as follows: The amino acids are counted in the sequences or sequence sections to be compared that are either identical or similar at the respective position. This number is divided by the total number of the amino acid residues and multiplied by 100. This yields a percentage of the sequence similarity or homology. This is illustrated by the sample given below:

TWALFKALSHMLCIGYGKFPPQS
PDAFWWAVVTMTTVGYGDMTPVG

The total number of the positions to be compared with one another is 23 residues; there are 7 identically and 6 similarily occupied amino acid positions. That is why the homology (7 + 6)/23 x 100 = 56.5%. An exchange of similar amino acids is also designated as a conservative exchange (cf. Dayhoff et al., 1978).

Insert 1

According to claim 1 there is provided a nucleic acid which comprises at least a part of the sequence of an I_h ion channel. The nucleic acid complementary thereto is also regarded as an inventive embodiment. Said nucleic acid may preferably be derived from a human DNA and is then in particular characterized by the sequences according to SEQ ID NO 1, SEQ ID NO 10, SEQ ID NO 11 and SEQ ID NO 15.

Advantageously, the sequence may also be derived from a rat DNA and is then in particular characterized by the SEQ ID NO 2 and SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 13 and SEQ ID NO 14.

In a further preferred embodiment, the sequence may be derived from a bovine DNA and is then characterized by the sequences according to SEQ ID NO 3 and SEQ ID NO 6, SEQ ID NO 7 and SEQ ID NO 12.

Furthermore, the sequence may preferably be derived from a sea urchin DNA, and it is then preferably characterized by the sequence SEQ ID NO 4.

Furthermore, the DNA may preferably be derived from *Drosphophila melanogaster*. The complete sequence is then in accordance with SEQ ID NO 5.

Insert 1:

The above isolated or purified nucleic acid molecules also can be characterized in terms of "percentage of sequence identity." In this regard, a given nucleic acid molecule as described above can be compared to a nucleic acid molecule encoding a corresponding gene (i.e., the reference sequence) by optimally aligning the nucleic acid sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence, which does not comprise additions or deletions, for optimal alignment of the two sequences. The percentage of sequence identity is calculated by determining the number of positions at which the identical nucleic acid base occurs in both sequences, i.e., the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by computerized implementations of known algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI, or BlastN and BlastX available from the National Center for Biotechnology Information, Bethesda, MD), or by inspection. Sequences are typically compared using BESTFIT or BlastN with default parameters.

"Substantial sequence identity" means that at least 75%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% of the sequence of a given nucleic acid molecule is identical to a given reference sequence. Typically, two polypeptides are considered to be substantially similar if at least 40%, preferably at least 60%, more preferably at least 90%, and most preferably at least 95% of the amino acids of which the polypeptides are comprised are identical to or represent conservative substitutions of the amino acids of a given reference sequence.

One of ordinary skill in the art will appreciate, however, that two polynucleotide sequences can be substantially different at the nucleic acid level, yet encode substantially similar, if not identical, amino acid sequences, due to the

degeneracy of the genetic code. The present invention is intended to encompass such polynucleotide sequences.

A particularly preferred embodiment comprises sequences that exhibit a homology of at least 80% to one of the sequences with the SEQ ID NO 1 to 15. In a further preferred embodiment the sequence exhibits a homology of at least 90% to one of the sequences designated by SEQ ID NO 1 to 15.

It hybridizes in a particularly preferred manner under low stringent conditions and even more preferably under conditions of high stringency with one of the sequences designated by SEQ ID NO 1 to 15.

The present invention covers modifications of the sequences according to SEQ ID NO 1 to 15 which result e.g. from the degeneration of the genetic code, deletions, insertions, inversions and further mutations, the biological property of the encoded channel protein or part thereof being preferably maintained.

Furthermore, the invention relates to an mRNA molecule comprising a sequence corresponding to one of the above-described sequences. Accordingly the invention covers a polypeptide which is encoded by the above-mentioned nucleic acid.

Insert 2

The above-described sequences can be used for a screening method or also a diagnosing method. In a screening method, it is possible owing to the identification of the sequence of the I_h channel to test the effect of substances on ion channels using said sequences.

Such a screening method may e.g. comprise the following steps:

Insert 2:

A nucleic acid molecule as described above can be cloned into any suitable vector. The selection of vectors and methods to construct them are commonly known to persons of ordinary skill in the art and are described in general technical references (see, in general, "Recombinant DNA Part D," *Methods in Enzymology*, Vol. 153, Wu and Grossman, eds., Academic Press (1987); Birren et al., *Genome Analysis: A Laboratory Manual Series, Volume 1, Analyzing DNA*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1997); Birren et al., *Genome Analysis: A Laboratory Manual Series, Volume 2, Detecting Genes*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998); Birren et al., *Genome Analysis: A Laboratory Manual Series, Volume 3, Cloning Systems*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1999); Birren et al., *Genome Analysis: A Laboratory Manual Series, Volume 4, Mapping Genomes*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1999); and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). Desirably, the vector comprises regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of host into which the vector is to be introduced, as appropriate and taking into consideration whether the vector is DNA or RNA. Preferably, the vector comprises regulatory sequences that are specific to the genus of the host. Most preferably, the vector comprises regulatory sequences that are specific to the species of the host.

Constructs of vectors, which are circular or linear, can be prepared to contain an entire nucleic acid sequence as described above or a portion thereof ligated to a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived from ColE1, 2 mμ plasmid, λ, SV40, bovine papilloma virus, and the like.

In addition to the replication system and the inserted nucleic acid, the

construct can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to antibiotics, heavy metals, etc., complementation in an auxotrophic host to provide prototrophy, and the like.

Suitable vectors include those designed for propagation and expansion or for expression or both. A preferred cloning vector is selected from the group consisting of the pUC, series the pBluescript series (Stratagene, LaJolla, CA), the pET series (Novagen, Madison, WI), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clonetech, Palo Alto, CA). Bacteriophage vectors, such as λGT10, λGT11, λZapII (Stratagene), λ EMBL4, and λ NM1149, also can be used. Examples of plant expression vectors include pBI101, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clonetech, Palo Alto, CA). Examples of animal expression vectors include pEUK-C1, pMAM and pMAMneo (Clonetech).

An expression vector can comprise a native or nonnative promoter operably linked to an isolated or purified nucleic acid molecule as described above. The selection of promoters, e.g., strong, weak, inducible, tissue-specific and developmental-specific, is within the skill in the art. Similarly, the combining of a nucleic acid molecule as described above with a promoter is also within the skill in the art.

Thus, in view of the above, the present invention also provides a host cell comprising an isolated or purified nucleic acid molecule or a vector as described above. Examples of host cells include, but are not limited to, a human cell, a human cell line, *E. coli, B. subtilis, P. aerugenosa, S. cerevisiae,* and *N. crassa.* Other examples include *E. coli* TB-1, TG-2, DH5α, XL-Blue MRF' (Stratagene), SA2821 and Y1090.

- producing homogeneous channel preparations, for example, by expression of the above-mentioned nucleic acid in a suitable host, such as oocytes, mammalian cells, etc.,

- testing of substances on said channel preparations.

It can here be determined by measuring the channel activity under the action or in the absence of test substances which substances are suited for influencing the channels.

The invention also relates to a kit for performing such a screening method which comprises at least one of the above-described nucleic acids or polypeptides.

The sequences can also be used for a diagnosing method, in particular for recognizing cardiovascular disorders.

In said diagnosing method the nucleic acid of the patient is preferably contacted with a sequence section of one of the above-described DNAs and/or RNAs, whereby a signal is obtained that is indicative of the presence and/or absence of an ion-channel nucleic acid sequence. Mutations in the ion channels of the patient can also be detected by selecting suitable samples, e.g. short oligonucleotides, which in turn is of help to the differential diagnosis.

Furthermore, the present invention refers to a kit for carrying out such a diagnosing method comprising one of the above-described sequences.

Furthermore, it is possible to use the above-described sequences for the treatment and/or prophylaxis of cardiovascular disorders and disturbances of consciousness as well as pain states. In a preferred embodiment, cardiovascular disorders that are due to

a faulty control of the sinus node can be treated or recognized at an early stage. Furthermore, disturbances of consciousness that are due to a malfunction of cortico-thalamic neurons are preferably recognized. For instance, within the scope of gene therapy, a fully operable ion channel as encoded by the nucleic acids described herein are introduced into a patient to replace a channel that is no longer operative.

Insert 3

Lastly, the invention relates to a pharmaceutical composition which comprises one or more of the above-described nucleic acids or the above-described polypeptide. Such a pharmaceutical composition can be used for treating cardiovascular disorders, in particular those that are due to a faulty control of the sine node, as well as disturbances of consciousness, in particular those caused by a malfunction in cortico-thalamic neurons.

Insert 4

The invention shall now be described with reference to the examples and the attached figures, of which:

Figure 1A shows the nucleic-acid and the derived protein sequence of the channel from sea urchin *Strongylocentrotus purpuratus* (SPHI channel).

Figure 1B shows the S4 motif of said channel protein, as compared with other known channel sequences;

Figure 1C shows the pore motif of said sequence as compared with other sequences of other channels;

Figure 1D shows the cNMP-binding domain of the cDNA of the I_h ion channel as compared with other sequences of ion channels;

Insert 3:

Accordingly, the present invention provides a method of prophylactically or therapeutically treating a mammal for a cardiovascular disorder, in particular a cardiovascular disorder that is due to a faulty control of the sinus node. The method comprises administering to a mammal (i) a vector comprising and expressing a prophylactically or therapeutically effective amount of an above-described nucleic acid or (ii) a prophylactically or therapeutically effective amount of an above-described polypeptide, whereupon the mammal is treated for the cardiovascular disorder.

The present invention further provides a method of prophylactically or therapeutically treating a mammal for a disturbance of consciousness, in particular a disturbance of consciousness that is due to a malfunction in thalamic neurons. The method comprises administering to a mammal (i) a vector comprising and expressing a prophylactically or therapeutically effective amount of an above-described nucleic acid or (ii) a prophylactically or therapeutically effective amount of an above-described polypeptide, whereupon the mammal is treated for the disturbance of consciousness.

Still further provided by the present invention is a method of prophylactically or therapeutically treating a mammal for a pain state. The method comprises administering to a mammal (i) a vector comprising and expressing a prophylactically or therapeutically effective amount of an above-described nucleic acid or (ii) a prophylactically or therapeutically effective amount of an above-described polypeptide, whereupon the mammal is treated for the pain state.

Insert 4:

Therefore, the present invention also provides a composition comprising an above-described isolated or purified nucleic acid (or vector comprising the nucleic acid) or an above-described polypeptide and a carrier therefor. Carriers, such as pharmaceutically acceptable carriers, are well-known in the art, and are readily available. The choice of carrier will be determined in part by the particular route of administration and whether a nucleic acid molecule or a polypeptide moleculeis being administered. Accordingly, there is a wide variety of suitable formulations for use in the context of the present invention, and the invention expressly provide a pharmaceutical composition that comprises an active agent of the invention and a pharmaceutically acceptable carrier therefor. The following methods and carriers are merely exemplary and are in no way limiting.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluent, such as water, saline, or orange juice; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth. Pastilles can comprise the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to the active ingredient, such excipients/carriers as are known in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The

formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Further suitable formulations are found in Remington's Pharmaceutical Sciences, 17th ed., (Mack Publishing Company, Philadelphia, Pa.: 1985), and methods of drug delivery are reviewed in, for example, Langer, Science, 249, 1527-1533 (1990).

Generally, when an above-described polypeptide is administered to an animal, such as a mammal, in particular a human, it is preferable that the polypeptide is administered in a dose of from about 1 to about 1,000 micrograms of the polypeptide per kg of the body weight of the host per day when given parenterally. However, this dosage range is merely preferred, and higher or lower doses may be chosen in appropriate circumstances. For instance, the actual dose and schedule can vary depending on whether the composition is administered in combination with other pharmaceutical compositions, or depending on interindividual differences in pharmacokinetics, drug disposition, and metabolism. One skilled in the art easily can make any necessary adjustments in accordance with the necessities of the particular situation.

If desired, the half-life of the polypeptide can be increased by conjugation to soluble macromolecules, such as polysaccharides, or synthetic polymers, such as polyethylene glycol, as described, for instance, in U.S. Patents 5,116,964, 5,336,603, and 5,428,130. Alternately, the polypeptides can be "protected" in vesicles composed of substances such as proteins, lipids (for example, liposomes), carbohydrates, or synthetic polymers. If liposomes are employed, liposome delivery can be carried out as described in U.S. Patent 5,468,481, or using liposomes having increased transfer capacity and/or reduced toxicity *in vivo* (see, e.g., PCT patent application WO 95/21259 and the references cited therein). Furthermore, polypeptides can be administered in conjunction with adenovirus (preferably replication-deficient adenovirus) to allow the intracellular uptake of the polypeptides by adenoviral-mediated uptake of bystander molecules (e.g.,

as described in PCT patent application WO 95/21259). Similarly, a conjugate, such as one comprising a targeting moiety, or a fusion of a an above-described polypeptide to an antibody (or an antigenically reactive fragment thereof) that recognizes a cell surface antigen; etc. can be employed to deliver the resultant fusion protein to a specific target cell or tissue (e.g., as described in U.S. Patent 5,314,995).

Those of ordinary skill in the art can easily make a determination of the vector to be administered to an animal, such as a mammal, in particular a human. The dosage will depend upon the particular method of administration, including any vector or promoter utilized. For purposes of considering the dose in terms of particle units (pu), also referred to as viral particles, it can be assumed that there are 100 particles/pfu (e.g., $1x10^{12}$ pfu is equivalent to $1x10^{14}$ pu). An amount of recombinant virus, recombinant DNA vector or RNA genome sufficient to achieve a tissue concentration of about 10^2 to about 10^{12} particles per ml is preferred, especially of about 10^6 to about 10^{10} particles per ml. In certain applications, multiple daily doses are preferred. Moreover, the number of doses will vary depending on the means of delivery and the particular recombinant virus, recombinant DNA vector or RNA genome administered.

Further provided by the present invention is a hybridoma cell line that produces a monoclonal antibody that is specific for an above-described isolated or purified polypeptide molecule. Methods of making hybridomas are known in the art (see, e.g., Harlow et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988); Harlow et al., *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1999)). Thus, the present invention also provides the monoclonal antibody produced by the hybridoma cell line. Similarly, the present invention provides a polyclonal antiserum raised against an above-described isolated or purified polypeptide molecule. Methods of raising polyclonal antiserum against a polypeptide molecule are also known in the art (see, e.g., Harlow et al.(1988), *supra*; Harlow et al.(1999), *supra*).

Figure 2A shows the inward current having a complex waveform, which is triggered by the hyperpolarizing voltage steps from a holding voltage of +10 mV to more negative test values;

Figure 2B shows the equilibrium current/voltage (I/V) relationship determined at the end of a hyperpolarizing voltage pulse;

Figure 2C shows the measuring protocol for the determination of the "instantaneous" I/V relationship from the amplitude of the tail currents;

Figure 2D shows the "instantaneous" I/V relationship which is slightly outwardly rectifying, at a reversal voltage V_{rev} of –30 mV;

Figure 2E shows that the time course of the "tail" currents depends on the time of the change in voltage;

Figure 2F shows the voltage dependence of the relative probability that the channel is open, P_o, which was determined from the amplitude of tail currents at +10 mV, similar to those illustrated in Fig. 2A;

Figure 3A shows the induction of large whole-cell currents by hyperpolarization in the presence of 1 mM cAMP, which currents developed with a delay and slowly reached an equilibrium;

Figure 3B shows the voltage dependence of P_o, determined from normalized whole-cell "tail" currents and "tail" currents of inside-out patches;

Figure 3C shows the rapid rise in amplitude of the inward current after short UV exposure;

Figure 3D shows SPIH currents of cell-free membrane pieces without cAMP; and

Figure 3E shows the same as Fig. 3D, but with cAMP;

Figure 3F shows the dependence of the current on the cAMP concentration which can be described by a simple binding isotherm with $K_{1/2}$ of 0.74 µM and a Hill coefficient which does not clearly differ from one;

Figures 4A and 4B show the blockade of the SPIH channels by Cs^+ (Figure 4A control, Figures 4B + 10 mM Cs^+);

Figure 4C shows the I/V relationship in the presence of 0 to 10 mM Cs^+;

Figure 4D shows a plot of standardized current I/I_{max} (at –70 mV) against Cs^+;

Figure 4E shows the ion selectivity of SPIH channels on inside-out patches, in the case of which 100 mM of the bath K^+ were replaced by corresponding concentrations of Rb^+, Na^+, Li^+ or Cs^+;

Figure 4F shows the I/V relationship under the various ionic conditions shown in part E;

Figure 4G shows that the inward currents were interrupted almost entirely, whereas the amplitudes of the outward currents did not change when the extracellular medium just contained Na^+;

Figure 4H shows the I/V relationship of the currents from part G at different K^+ concentrations;

Figure 5A shows a Northern Blot of the channel messenger RNA with a major transcript of about 3.3 kb and a minor transcript of 2.9 kb;

Figure 5B is a light-microscopic photograph of sperms from *S. purpuratus* (right picture) and the corresponding immunohistochemical staining with an antibody which specifically recognizes the SPIH channel (left picture).

Figure 5C shows a corresponding Western Blot analysis.

Figure 6 is a schematic illustration showing the pc SPIH construct that was used for the heterologous expression of SPIH in HEK 293 cells. The cDNA region is illustrated as a hatched bar; the adjoining regions of the plasmid vector (pcDNA I) as bold lines. The orientation of the cDNA in the plasmid vector can be inferred from the position of the promoter for the T7 polymerase and the restriction sites in the multiple cloning region. The inserted Kozak sequence is designated by K.

A typical representative of an ion channel protein according to the invention is the channel from sea urchin (SPIH). The channel activity of HEK 293 cells, which had been transfected with the pcSPHI construct (Fig. 6), was examined with the help of the patch-clamp method in the whole-cell configuration. Hyperpolarizing voltage steps showed an inward current with a complex waveform (cf. Fig. 2A). A fast current component that was not time-resolved was followed by a time-dependent current that developed with a delay and, after the maximum had been reached, decreased into smaller amplitudes when the test voltage was $V_m \leq -30$ mV (Fig. 2A). After V_m had been set back to +10 mV, "tail" currents developed that also showed a complex time course. The steady-state

relationship between current/voltage (I/V), at the end of the hyperpolarizing voltage pulse (arrow in Fig. 2A), showed a strong inward rectification (Fig. 2B). The "instantaneous" I/V relationship was determined from the amplitude of the tail currents using a different protocol for the voltage steps (Fig. 2C). The "instantaneous" I/V relationship was slightly outwardly rectifying with a reversal voltage, V_{rev}, of –30 mV (Fig. 2D). The I/V relationship became approximately linear at higher $[K^+]_o$ because the inward sodium current was significantly amplified by $[K^+]_o$ (see Fig. 4H). The conclusion can be drawn that the currents are strongly inwardly rectifying because the SPIH channel at positive voltages is either closed or inactivated. The voltage dependence of the open probability, P_o (Fig. 2F), was determined from the amplitude of the tail currents at +10 mV (Fig. 2A). The voltage, $V_{1/2}$, at which a half-maximal current was observed, was at –26.1 mV (7 experiments). Thus the SPIH channel is inactive at voltages $\geq$ +10 mV and is opened by hyperpolarization. This voltage dependence reminds of hyperpolarization-activated currents (I_h) which occur in different cells (DiFrancesco, 1990, 1993; Pape, 1996). Because of its unusual properties, the I_h has also been designated as a "queer" or "funny" current (I_q and I_f). The channel according to the invention is (1) activated at hyperpolarizing voltages; (2) directly modulated by cyclic nucleotides; (3) blocked by millimolar concentrations of extracellular Cs^+; (4) it is cation-selective at a P_{Na}/P_K of ~ 0.2 to 0.4; and (5) the inward sodium currents are sensitive to $[K^+]_o$. The following experiments demonstrate that said features are also found in the heterologously expressed SPIH channel.

With 1 mM cAMP in the pipette solution, hyperpolarization produced large currents which developed with a delay and slowly reached a steady state (Fig. 3A).The sigmoidal time course of the current (see Fig. 3A, box) is characteristic of the time course of vertebrate I_h currents. 1 mM cGMP in the pipette also changed the SPIH-induced currents. The voltage dependence of P_o was determined with the help of whole-cell tail currents (Fig. 3B). A fit to the Boltzmann equation yielded $V_{1/2}$ = -50.8 mV. The dialysis of the cell with

the pipette solution took several minutes; thus transient effects of cAMP might impair the test. A technique was therefore employed using the rapid photorelease of cAMP or cGMP from "caged" derivatives (cf. Adams and Tsien, 1993; Hagen et al., 1996). The cells were dialyzed with 100 μm "caged" cAMP and the SPIH channels were activated by changing the V_m from +10 mV to -70mV; a short flash of UV light then effected a rapid increase in the amplitude of the SPIH-induced inward current (Fig. 3C). The hyperpolarization-activated currents before the flash resembled control currents (Fig. 3C, trace 1), while amplitude and time course of the currents after the UV flash (Fig. 3C, trace 2) were similar to those recorded in the presence of cAMP (Fig. 3E). With 100 μM "caged" cGMP in the pipette, UV flashes of similar duration and similar intensity did not change the SPIH-induced currents. A binding motif for cyclic nucleotides suggests that cAMP could directly enhance the channel activity without the participation of a phosphorylation mechanism. To verify this hypothesis the SPIH currents were measured on excised membrane patches without (Fig. 3D) and with cAMP (Fig. 3E). cAMP (1mM) enhanced the amplitudes of the voltage-activated currents by up to 20-fold. The increase in current by cAMP was reversible and did not require Mg^{2+}/ATP. The superfusion of the excised membrane patches with solutions containing different cAMP concentrations enhanced the SPIH currents in a dose-dependent way. The dependence of the current on the cAMP concentration can be described by a simple binding isotherm with a $K_{1/2}$ of 0.74 μM and a Hill coefficient which does not significantly differ from one (Fig. 3F). In the separated membrane patches, $V_{1/2}$ in the presence of cAMP was about 35 mV more negative than $V_{1/2}$ measured in the whole-cell configuration (Fig. 3B). This observation might suggest that an endogenous factor provided by the HEK293 cell also determines $V_{1/2}$. cGMP concentrations of up to 1mM did not change the amplitude of the SPIH currents. The conclusion can be drawn from this experiment that cAMP, but not cGMP, can modulate the SPIH channel activity. Thus, in contrast to CNG channels (Finn et al., 1996) SPIH is under the double control of voltage and cAMP. Blockage of the SPIH channels by extracellular Cs^+ was examined on "outside-out" membranes with the

voltage protocol of Fig. 2C. Cs^+ blocked the SPIH channel in a concentration- and voltage-dependent manner. In the presence of 10 mM Cs^+ the inward currents disappeared completely, whereas outward tail currents were still present (cf. Figs. 4A and 4B). The I/V relationship in the presence of from 0 to 10 mM Cs^+ is shown in Fig. 4C. The standardized current I/I_{max} (at –70 mV) was plotted against $[Cs^+]$ (Fig. 4D). The data were fitted with an inhibitory constant K_i of 245 µM and a Hill coefficient of n = 1.2. The ion selectivity of the SPIH channel was determined with inside-out membranes. The bath solutions always contained 0.1 mM cAMP to increase the amplitude of the currents. 100 mM K^+ in the bath were replaced by Rb^+, Na^+, Li^+ or Cs^+ (Fig. 4E). The permeability ratios $P_K : P_{Rb} : P_{Na} : P_{Li} : P_{Cs}$ were calculated as 1 : 0.7 : 0.26 : 0.15 : 0.06. The ion selectivity of SPIH concurs well with the ion selectivity of various vertebrate I_h channels (Pape, 1996; Wollmuth and Hille, 1992). When the extracellular medium only contained Na^+, the inward currents were eliminated almost entirely, whereas the amplitudes of the outward currents did not change significantly (Fig. 4G). Elevation of $[K^+]_o$ to 5 and 20 mM dramatically increased the inward currents. These results demonstrate that the SPIH channel conducts little, if any, sodium in the absence of potassium ions.

The expression of messenger RNA of the channel protein was analyzed by means of Northern Blots. A major transcript of around 3.3 kb and a minor transcript of 2.9 kb were detected in poly(A)$^+$RNA of male, but not female, gonads (Fig. 5A). The size of the transcripts concurs well with the size of the cloned cDNA (3 kb). The SPIH-specific probe did not hybridize with poly(A)$^+$RNA isolated from the intestine of sea urchin (Fig. 5A). The exclusive expression of SPIH mRNA in male gonads suggests that the channel is expressed in sperms. This hypothesis was tested with purified antibodies FPc44K and FPc45K directed against a fusion protein of the C-terminal domain of the channel polypeptide (residues 662-767). The antibodies were used for Western Blot analyses (Fig. 5C) and immunocytochemistry (Fig. 5B). Both antibodies recognized a main band of M_r ~ 92K in Western Blots of flagellar membranes which had been purified from sea

urchin sperm (Fig. 5C, lane 3). Membranes which had been purified from the sperm head were not recognized by the antibodies (Fig. 5C, lane 5). This result was confirmed by immunocytochemistry with individual sperms. The antibody FPc45K almost exclusively stained the sperm flagellum (Fig. 5B); the weak staining of some head structures presumably represents unspecific cross reactivity of the antibody. A band of M_r ~ 88K was observed in Western Blots of membranes of transfected HEK293 cells (Fig. 5C, lane 2). The M_r of the channel polypeptide, expressed in HEK293 cells, is almost identical with the M_r value as is to be expected of the derived amino acid sequence (87.9K). In membranes of non-transfected HEK293 cells, no 88K polypeptide was detected by the antibody (Fig. 5C, lane 1). The treatment of flagellar membranes with alkaline phosphatase lowered the M_r of the native polypeptide from ~ 92 K to 88K. Since native and heterologously expressed polypeptides were of a similar size, the cloned cDNA carries the complete coding sequence of SPIH. The small decrease in M_r under dephosphorylating conditions demonstrates that the native polypeptide in phosphorylated form is present with a slightly reduced electrophoretic mobility. In most dephosphorylation experiments the shift from 92K to 88K was not complete, and at least two intermediate bands were observed. This result suggests that the channel poplypeptide should be phosphorylated several times. The SPIH sequence carries sequence motifs for the phosphorylation by PKA, PKG, PKC and tyrosine kinase (see Fig. 1A).

The electrophysiological properties unequivocally identify SPIH as a member of the I_h channel family. However, we also noticed characteristic differences between SPIH and vertebrate I_h channels. First, in the absence of cAMP the SPIH current is transient, whereas in the presence of cAMP the time course is similar to that in vertebrate I_h channels. Second, the large augmentation of the SPIH current by cAMP primarily arises from an increase in the maximum current while cAMP modulates the cardiac I_h channel such that $V_{1/2}$ is shifted towards more positive values (DiFrancesco, 1993) without

influencing the maximum amplitudes (see, however, Ingram and Williams, 1996; Accili et al., 1997). Finally, the cardiac I_h is also modulated by micromolar cGMP concentrations (DiFrancesco and Tortora, 1991), whereas SPIH does not exhibit said effect. The SPIH channel is very similar to both the voltage-controlled K^+ channels and the CNG cation channels. That is why the I_h channels form a class of their own within the superfamily of the voltage-controlled channels. SPIH has a characteristic motif of a voltage sensor (S4) like the K^+, Na^+ and Ca^{2+} channels that are opened by depolarization. Although there is no a priori reason to rule out the S4 motif as a voltage sensor in a hyperpolarization-activated channel, the mechanism of an activation as in HERG-K^+ channels (Trudeau et al., 1995; Smith et al., 1996) is more likely. It has been demonstrated with respect to the strong inward rectification of HERG that it is the result of the inactivation which closes the channels at positive voltages, but the channels recover rapidly from the inactivation at negative voltages. In HERG channels the inactivation is much faster than the activation and is therefore not visible kinetically (Smith et al., 1996). Together with the CNG channels SPIH possesses a cyclic nucleotide-binding region, and its properties are modulated by cAMP. cAMP probably intensifies the SPIH activity by binding to the highly conserved cyclic nucleotide-binding region. In CNG channels, it has been demonstrated with respect to the high selectivity for cGMP that said selectivity is accompanied by a Thr residue (T363 in the α-subunit of the rod photoreceptor; Altenhofen et al., 1991) and an Asp residue (D604 in rCNGα; Varnum et al., 1995). The SPIH has Val and Ile residues at the corresponding positions; it is presumed that these positions also control the ligand selectivity in SPIH. The physiological importance of the I_h channels in flagellar membranes of sperm could be explained as follows: the stimulation of *S. purpuratus* sperm with the chemotatic peptide "speract" causes a hyperpolarization (Lee and Garbers, 1986; Garbers, 1989), of which it is assumed that it is due to the opening of a K^+ channel (Babcock et al., 1992). At higher peptide concentrations the hyperpolarization is followed by a depolarization (Babcock et al, 1992). Two (or more) ion channel types with different selectivity and pharmacology could contribute to the "speract"-induced

depolarization (see Darszon et al, 1996). One of said channels has a weak K^+ selectivity ($P_{Na/Pk} \cong 0.2$) and an extremely low P_o (at $V_m = 0$ mV) which is considerably enhanced by cAMP, but not by cGMP (Labarca et al., 1996). These observations suggest that said channel is actually SPIH. The "speract"-induced hyperpolarization could initiate the SPIH channel activity which then could even be augmented by a simultaneous increase in the cAMP level (Hansbrough et al., 1980) with the help of a voltage-dependent adenylate cyclase (Beltrán et al, 1996). At the given ionic composition of sea water and a P_{Na}/P_K of 0.2 to 0.4 the opening of the SPIH channel and the subsequent Na^+ influx could effect the "speract"-induced depolarization. It can also reasonably be assumed that the I_h channels, for instance in cardiac cells or thalamic neurons, take part in the generation of oscillations of the membrane voltage, thereby causing the oscillation of Ca^{2+} in the flagellum (Suarez et al, 1993). The change in $[Ca^{2+}]_i$, could change the flagellar beating, thereby contributing to the chemotactic response.

EXAMPLES

Methods

Isolation of the cDNA clones

With two degenerated primers (# 1764 and # 1772) a PCR was carried out on single-strand cDNA (from sea urchin gonads, *Drosophila melanogaster*, bovine retina, olfactory tissue of the rat) or on cDNA libraries (from human thalamus or heart). A 100 µl PCR batch had the following composition: 3-10 ng of first-strand cDNA and about 10^5 pfu of the cDNA libraries, respectively, 1.6 µg of the degenerated primer each, 1 x PCR buffer, 2mM dNTP, 1 U PrimeZyme (Biometra). The PCR batch was first denatured at 94°C for 2 min and then incubated for 45 cycles in the following manner:

denaturation:	94°C, 45 sec
hybridization:	48°C, 45 sec
polymerization:	72°C, 40 sec

The sequences of the degenerated primers are (in 5'→3' direction):

1764: CTGACTGCAGARGTNTTYCARCCNGGNGA (SEQ ID NO 16)

1772: ATCGGAATTCNCCRAARTANGANCCRTC (SEQ ID NO 17)

The PCR fragments amplified with the primers # 1764 and # 1772 were radiolabeled and used as probes for screening cDNA libraries under high stringency for the complete cDNAs. The partial clone HHIH (SEQ ID NO 11) was isolated by low-stringency hybridization. The hybridization conditions were as follows:

	high stringency	low stringency
prehybridization	5 x SSC[(1)], 5 x Denhardt's[(2)], 0.1% SDS, 0.1 mg/ml herring sperm DNA, 1-2 h, 65°C	5 x SSC[(1)], 5 x Denhardt's[(2)], 0.1% SDS, 0.1 mg/ml herring sperm DNA, 1-2 h, 55°C
hybridization	prehybridization solution with 50-100 ng ^{32}P-labeled DNA (1-10^6 cpm/ml), 12-14 h, 65°C	prehybridization solution with 50-100 ng ^{32}P-labeled DNA ($1x10^6$ cpm/ml), 12-14 h, 65°C
washing	1 x SSC(1), 0.1% SDS 2 x 30 min, 65°C	2 x SSC(1), 0.1% SDS 2 x 30 min, 55°C

(1) 1 x SSC 150 mM NaCl, 15 mM Na citrate, pH 7.0

(2) 1 x Denhardt's Ficoll, polyvinylpyrrolidone, bovine serum albumin (0.2 g/l each)

The positive phages were isolated and the cDNA was converted by "in vivo excision" (in case of λZAPII phages) into pBluescriptSK derivatives. The cDNA was excised with EcoRI from λgt11 phages and subcloned into pBluescriptSK plasmid DNA. The DNA was sequenced with the dideoxy-mediated chain termination technique (Sanger et al., 1997).

Northern and Western Blots

Poly(A)$^+$RNA, isolated from different sea urchin tissues, was analyzed by Northern blotting. Each lane contained about 10 µg poly(A)$^+$RNA. The blot was hybridized with a ^{32}P-labeled 1074 bp cDNA fragment (nucleotide positions) at 42°C, 5 x SSC and 50% formamide. A C-terminal region of the SPIH polypeptide was expressed as a fusion construct with the maltose binding protein. The purified fusion protein was used for producing the polyclonal antibodies FPc44K and FPc45K; the antibodies were purified from rabbit serum by affinity chromatography using the fusion protein. Sperm flagella were separated from the head according to Darszon et al. (1994). Purified flagella and

head membranes were homogenized in a solution buffer containing 150 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes at pH 7.5, 0.1 mM EGTA and 0.5% Triton X-100, followed by a centrifugation at 40,000 rpm for 60 minutes. This process was repeated two times. Transfected HEK293 cells were homogenized in a lysis buffer (10 mM Hepes, 1 mM DTT and 1 mM EDTA at pH 7.4), 5 x freeze-dried (in liquid N_2) and finally centrifuged at 55,000 rpm for 10 minutes. The membrane pellet was dissolved in the solution buffer. Flagellar membrane proteins were dephosphorylated with a unit of alkaline phosphatase in solution buffer at 30°C for 30 to 60 min. The membrane proteins were separated by SDS-PAGE, transferred to Immobilon membranes and labeled with the polyclonal antibodies. The immunoreactivity was made visible by the ECL detection kit (Amersham). Immunocytochemistry on an individual sperm was carried out as described above (Weiner 1997).

Electrophysiology

cDNA coding the SPIH polypeptide was transiently expressed in HEK293 cells, as described earlier (Baumann et al, 1994). SPIH-controlled currents were recorded with the patch-clamp method in the whole-cell configuration and cell-free membrane patches. The composition of various bath and pipette solutions is indicated in the legends of the figures (see below). The channels were activated by stepping the membrane voltage from +10 mV to various negative voltage values. Leakage currents were subtracted using a P/8 protocol. The voltage dependence of the probability that the channel is open was determined from tail currents at +10 mV. The blockade of the SPIH channel by Cs^+ was analyzed with outside-out membrane patches in the presence of 1 mM cAPM in a pipette solution. The solutions in the bath contained 0.03 to 10 mM CsCl. Relative ion permeabilities were calculated from the respective shift of V_{rev}, which was measured on cell-free inside-out membrane patches, when 100 mM K^+ in the bath had been replaced

by Na^+, Li^+, Rb^+ or Cs^+. Experiments with "caged" cAMP or "caged" cGMP were carried out as described earlier (Hagen et al. 1996).

The results of said experiments are now described in more detail.

Figure 1A shows the nucleic acid sequence and the derived amino acid sequence of the I_h channel of sea urchin (SPIH). Nucleotides are numbered in 5'→3' direction, +1 corresponding to the first nucleotide of the start codon (ATG) of the open reading frame. Nucleotides that are 5'-located from nucleotide +1 are designated by negative figures. The derived amino acid sequence (one-letter code) is indicated under the nucleic acid sequence and is also numbered. The start codon (ATG), the corresponding methionine and the stop codon (TGA; pos. 2302-2304) are printed in bold. Stop codons in the same reading frame before the start codon are underlined. The polyadenylation signal at position 2501-2507 is boxed. The position of the transmembranal segments S1-S6, of the pore-forming region and of the binding site for cyclic nucleotides (cNMP binding site) is marked by bars above the nucleic acid sequence. The limits of said regions are defined by sequence comparison with other voltage-dependent K^+ channels, EAG-K^+ channels and CNG channels. Consensus sequences for phosphorylation by cAMP/cGMP-dependent kinases are marked by triangles (Δ). Consensus sequences for phosphorylation by protein kinase C are marked by circles (•) and that by tyrosine kinase by an asterisk (✱). The SPIH sequence (SEQ ID NO 4) codes for a protein of 767 amino acids with a calculated molecular weight of 87,937 Da.

Figure 1B shows a comparison of the voltage-sensor (S4) motifs of the I_h channel of sea urchin and other channels. Regularly spaced Arg or Lys residues are boxed. Other positively charged residues are in bold.

Shaker (Pongs et al., 1988), K^+ channel encoded by the *Drosophila* Shaker gene;

DmEAG (Warmke et al, 1997), *Drosophila* EAG channel;
HERG, human EAG-related gene (Warmke and Ganetzky, 1994);
KAT1 (Anderson et al, 1992), K^+ channel of *Arabidopsis thaliana*;
brCNGCα (Kaupp et al, 1989), α-subunit of the cyclic nucleotide-controlled channel from bovine rod photoreceptors.

Figure 1C shows the pore motif of SPIH with the pore motifs of other members of the superfamily of the voltage- and cyclic nucleotide-controlled ion channels:

The residues which are identical or similar to the corresponding amino acids in SPIH are highlighted by a black or grey background.

Figure 1D shows a sequence comparison of cNMP binding domains.
boCNGCα, the α-subunit of the CNG channel of bovine olfactory neurons (Ludwig et al., 1990); PKA1, the cAMP binding site 1 of the protein kinase A (Titani et al., 1984); the cGMP binding site 1 of the protein kinase G (Takio et al, 1984); CAP, the catabolite activator protein (Aiba et al., 1982). Residues that are highly conserved in cyclic nucleotide-binding motifs are indicated by arrows; residues that determine the ligand selectivity in brCNGCα are indicated by an asterisk. Secondary-structure predictions derived from the cAMP binding domain of CAP are shown as bars below the sequence.

Figure 2 shows the electrophysiological characterization of the SPIH channel.

Figure 2A shows the current, which was recorded by transfected HEK293 cells in the whole-cell configuration. The current was activated by stepping the voltage from a holding value at +10 mV to various test values of -100 mV to +10 mV in increments of 10 mV. Tail currents were recorded by stepping the voltage of the test value back to +10 mV. The HEK293 cells were flushed with a bath solution containing the following (mM):

135 NaCl, 5 KCl, 1.8 $CaCl_2$, 2.8 $MgCl_2$ and 5 Hepes-NaOH at pH 7.4; the pipette solution contained the following substances (mM): 126 KCl, 10 Hepes-KOH, 10 EGTA at pH 7.4.

In **Figure 2B,** there is plotted the voltage-current (I/V) relationship measured under equilibrium conditions at the time indicated by the arrowhead in Figure 2A.

Figure 2C shows the measurement protocol with which the "instantaneous" I/V relationship was determined; the voltage was first stepped from a holding value of 0 mV to –70 mV, followed by steps to test values in the range of from +50 mV to –70 mV in 10 mV increments.

Figure 2D then shows the plot of the "instantaneous" I/V relationship measured at the time indicated by the arrow in Figure 2C (inset).

Figure 2E shows that the time course of the "tail" currents depends on the time at which the voltage is reset to + 30 mV.

Figure 2F shows the voltage dependence of the relative open probability, P_o, of the channel. The tail current amplitudes (arrow in part a) were normalized to the maximum current. The midpoint voltage, $V_{1/2}$, was -26.1 mV. The effective charge amount, Q, which is flowing during channel switching, is 3.5 elementary charges. It was achieved from a fit of the Boltzmann function to the data: Mean of 7 experiments.

Figure 3 indicates the modulation of SPIH channels by cyclic nucleotides.

Figure 3A shows the whole-cell SPIH current in the presence of 1 mM cAMP. The voltage-step protocol is the same as in Fig. 2A. The bath contained (mM): 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 2.8 $MgCl_2$ and 5 Hepes-NaOH at pH 7.4; the pipette solution contained

(mM): 126 KCl, 10 Hepes-KOH, 10 EGTA at pH 7.4, and 1mM cAMP. The inset shows a magnification by way of which the sigmoidal time course can be seen particularly well.

Figure 3B shows the voltage dependence of the relative P_o, derived from normalized whole-cell tail currents at +10mV (●) and of tail currents recorded by inside-out patches (Δ). A continuous line represents a fit of the Boltzmann equation to the data. $V_{1/2}$ for the whole-cell currents of part A was –50.8mV and for the inside-out-patch currents of part E it was –84.7 mV; the Q values were 3.8 and 2.7, respectively.

Figure 3C shows the modulation of whole-cell SPIH currents by the photolysis of "caged" cAMP. The pipette solution contained 100 µM "caged" cAMP. The SPIH current was activated by voltage jumps from +10 mV to –70 mV before the UV flash was induced (trace 1) and after three consecutive UV flashes (trace 2). The time course of the flash-induced increase in current at –70 mV is shown below.

Figures 3D and E show voltage-activated SPIH currents in inside-out membrane patches without cAMP (D) and in the presence of 1 mM cAMP (E) in the bath. The voltage step protocol was carried out in the way as shown in Figure 2A. The pipettes and bath solutions contained (mM): 126 KCl, 10 Hepes-KOH, 10 EGTA at pH 7.4 and 1 mM cAMP (bath).

Figure 3F discloses the dependence of the SPIH current amplitude on the cAMP concentration; the cAMP concentrations were as follows (µM): 0.1; 0.3; 1; 3; 10 and 1000. A continuous line shows a fit of the Hill equation to the data; $K_{1/2}$ = 0.74 µM; n = 1.05; mean of 10 experiments.

Figure 4 shows several pharmacological properties of the SPIH channel.

Figures 4A and B show voltage-activated SPIH currents, recorded by outside-out membrane patches without (A) and with 10 mM Cs^+ (B) in the bath; the pipette solution contained the following (mM): 124 KCl, 10 Hepes-KOH, 10 EGTA at pH 7.4 and 1 mM cAMP; the bath solution contained (mM): 126 KCl, 10 Hepes-KOH, 10 EGTA at pH 7.4 and the illustrated concentrations of CsCl.

Figure 4C shows again the I/V relationship in the presence of 0 to 10 mM Cs^+ in the bath.

Figure 4D discloses the dependence of the normalized current at –70 mV on $[Cs^+]$. The continuous line shows a fit of the Hill equation to said data; K_i = 245 µM, Hill coefficient 1.2 (mean of 1-6 experiments).

Figure 4E shows the ion selectivity of the SPIH channel. V_{rev} was determined on inside-out patches by stepping the holding voltage (-70 mV) to test values between –30 mV and + 30 mV in 5 mV increments. The pipette solution contained the following (mM): 150 KCl, 10 Hepes-NMDG, 10 EGTA at pH 7.4; the bath solution was composed as follows (mM): 50 KCl, 100 XCl, 10 Hepes-NMDG, 10 EGTA at pH 7.4 and 0.1 cAMP.

Figure 4F shows the I/V relationship of the currents shown in part E. V_{rev} was 16.9 mV (Na^+), 20.6 mV (Li^+), 5.6 mV (Rb^+), and 24.6 mV (Cs^+); mean of 3 to 10 experiments. The relative ion permeabilities P_X/P_K were calculated according to the equation $P_X/P_K = \{[K^+]_o - [K^+]_j \exp(zFV_{rev}/RT)\} / [X^+]_i \exp(zFV_{rev}/RT)$.

Figure 4G shows the K^+ dependence of whole-cell inward Na^+ currents in the presence of 0.5 mM and 20 mM K^+ in extracellular medium.

Figure 4H shows the "instantaneous" I/V relationship in the presence of 0, 1, 3, 5, 10, and 20 mM K^+ in the bath.

The pipette solution was the same as in part B, the bath solution as in Figure 1A with the indicated K^+ concentrations; the ion intensities were adjusted to the same value by the respective NMDG concentrations.

Figure 5 shows the expression pattern of SPIH.

Figure 5A is a Northern Blot analysis of the tissue distribution of SPIH transcripts in mRNA of male gonads (lane 1), female gonads (lane 2) and intestinal cells (lane 3); 10 µl poly(A)$^+$RNA each.

Figure 5B is a Western Blot analysis of membranes of mock-transfected HEK293 cells (lane 1; 2.5 µg protein), HEK293 cells which were transfected with SPIH cDNA (lane 2; 2.5 µg protein), purified flagella from sperm of *S. purpuratus* (lane 3; 6 µg protein), dephosphorylated flagellar membranes (lane 4; 6 µg protein) and sperm heads (lane 5; 15 µg protein).

Reference table of the DNA sequences described in the text by SEQ ID numbers

SEQ ID NO	DNA sequence
1	Partial sequence of the I_h channel from human thalamus tissue
2	partial sequence of an I_h channel from olfactory rat tissue
3	partial sequence of an I_h channel from retinal bovine tissue
4	complete sequence of the I_h channel from sea urchin sperm
5	complete sequence of the I_h channel from *Drosophila melanogaster*
6	partial sequence of an I_h channel from retinal bovine tissue
7	partial sequence of an I_h channel from retinal bovine tissue
8	partial sequence of an I_h channel from olfactory rat tissue
9	partial sequence of an I_h channel from olfactory rat tissue
10	partial sequence of an I_h channel from human thalamus tissue
11	partial sequence of an I_h channel from human heart tissue
12	complete sequence of an I_h channel from retinal bovine tissue
13	partial sequence of an I_h channel from olfactory rat tissue
14	partial sequence of an I_h channel from olfactory rat tissue
15	complete sequence of an I_h channel from human heart tissue

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WHAT IS CLAIMED IS:

1. An isolated or purified nucleic acid which codes for an I_h ion channel or a part thereof, or a nucleic acid complementary thereto, except for the nucleic acids with the sequences indicated in the GenBank database under the accession number AF028737 and in the ENHUM database under the accession number N72770, wherein said nucleic acid is DNA or RNA.

2. The isolated or purified nucleic acid of claim 1, characterized in that the nucleic acid is of human origin.

3. The isolated or purified nucleic acid of claim 2, characterized in that the nucleic acid comprises the sequence according to SEQ ID NO 1 or a part thereof.

4. The isolated or purified nucleic acid of claim 1, characterized in that the nucleic acid is of rat origin.

5. The isolated or purified nucleic acid of claim 4, characterized in that the nucleic acid comprises the sequence according to SEQ ID NO 2 or a part thereof.

6. The isolated or purified nucleic acid of claim 1, characterized in that the nucleic acid is of bovine origin.

7. The isolated or purified nucleic acid of claim 6, characterized in that the nucleic acid comprises a sequence according to SEQ ID NO 3 or SEQ ID NO 12 or a part of either of the foregoing sequences.

8. The isolated or purified nucleic acid of claim 1, characterized in that the nucleic acid is of sea urchin origin.

9. The isolated or purified nucleic acid of claim 8, characterized in that the nucleic acid comprises the sequence according to SEQ ID NO 4 or a part thereof.

10. The isolated or purified nucleic acid of claim 1, characterized in that the nucleic acid is of *Drosophila* origin.

11. The isolated or purified nucleic acid of claim 10, characterized in that the nucleic acid comprises the sequence according to SEQ ID NO 5 or a part thereof.

12. An isolated or purified nucleic acid characterized in that the sequence thereof is at least 80% identical to the isolated or purified nucleic acid of SEQ ID NO 1, 2, 3, 4 or 12.

13. The isolated or purified nucleic acid of claim 12, characterized in that the sequence thereof is at least 90% identical to the isolated or purified nucleic acid of SEQ ID NO 1, 2, 3, 4 or 12.

14. An isolated or purified nucleic acid characterized in that the nucleic acid hybridizes under low stringency conditions with SEQ ID NO 1, 2, 3, 4, 5 and/or 12.

15. The isolated or purified nucleic acid of claim 14, characterized in that the nucleic acid hybridizes under stringent conditions with SEQ ID NO 1, 2, 3, 4, 5 and/or 12.

16. A vector comprising the isolated or purified nucleic acid of claim 1.

17. A host cell comprising the vector of claim 16.

18 A composition comprising the isolated or purified nucleic acid of claim 1 and a carrier therefor.

19. An isolated or purified polypeptide encoded by a nucleic acid of claim 1.

20. An isolated or purified polypeptide encoded by a nucleic acid of claim 3.

21. An isolated or purified polypeptide encoded by a nucleic acid of claim 5.

22. An isolated or purified polypeptide encoded by a nucleic acid of claim 7.

23. An isolated or purified polypeptide encoded by a nucleic acid of claim 9.

24. An isolated or purified polypeptide encoded by a nucleic acid of claim 11.

25. An isolated or purified polypeptide encoded by a nucleic acid of claim 12.

26. An isolated or purified polypeptide encoded by a nucleic acid of claim 13.

27. An isolated or purified polypeptide encoded by a nucleic acid of claim 14.

28. An isolated or purified polypeptide encoded by a nucleic acid of claim 15.

29. A composition comprising the isolated or purified polypeptide of claim 19 and a carrier therefor.

30. A monoclonal antibody that specifically binds to the polypeptide of claim 19.

31. A method of screening a substance for the ability to influence the activity of an I_h ion channel, which method comprises:

(a) providing a homogeneous I_h ion channel preparation,

(b) contacting the homogeneous I_h ion channel preparation with the substance,

(c) measuring the activity of the I_h ion channel preparation in the presence of the substance, and

(d) comparing the activity of the I_h ion channel preparation in the presence of the substance with the activity of the I_h ion channel preparation in the absence of the substance, wherein a change in the activity of the I_h ion channel preparation in the presence of the substance as compared to the activity of the I_h ion channel preparation in the absence of the substance indicates that the substance can influence the I_h ion channel.

32. The method of claim 31, wherein said I_h ion channel preparation is prepared by expressing the isolated or purified nucleic acid which codes for an I_h ion channel or a part thereof, or a nucleic acid complementary thereto, except for the nucleic acids with the sequences indicated in the GenBank database under the accession number AF028737 and in the ENHUM database under the accession number N72770, wherein said nucleic acid is DNA or RNA, in a host cell.

33. The method of claim 31, wherein said I_h ion channel preparation consists essentially of the polypeptide encoded by the isolated or purified nucleic acid which codes for an I_h ion channel or a part thereof, or a nucleic acid complementary thereto, except for the

nucleic acids with the sequences indicated in the GenBank database under the accession number AF028737 and in the ENHUM database under the accession number N72770.

34. A method of diagnosing an I_h ion channel-associated disorder in a patient, which method comprises:

(a) contacting a nucleic acid sample from said patient with a detectably labeled isolated or purified nucleic acid of claim 1 under hybridizing conditions,

(b) detecting the label of the detectably labeled isolated or purified nucleic acid molecule, and

(c) comparing the level of detection of the label in (b) with the level of detection of the label in a control sample, wherein a difference in the level of detection of the label in (b) and the level of detection of the label in a control sample is indicative of an I_h ion channel-associated disorder in a patient.

35. The method of claim 34, wherein the detectably labeled isolated and purified nucleic acid is mutated, in which case the detection of the label in (b) is indicative of the presence of a nucleic acid encoding a mutated I_h ion channel in the nucleic acid sample of the patient.

36. The method of claim 34, wherein said I_h ion channel-associated disorder is a cardiovascular disorder.

37. A method of prophylactically or therapeutically treating a mammal for a cardiovascular disorder, which method comprises administering to said mammal a vector comprising and expressing a prophylactically or therapeutically effective amount of an isolated or purified nucleic acid of claim 1, whereupon said mammal is treated for said cardiovascular disorder.

38. The method of claim 37, wherein said cardiovascular disorder is due to a faulty control of the sinus node.

39. A method of prophylactically or therapeutically treating a mammal for a cardiovascular disorder, which method comprises administering to said mammal a prophylactically or therapeutically effective amount of a polypeptide of claim 19, whereupon said mammal is treated for said cardiovascular disorder.

40. The method of claim 39, wherein said cardiovascular disorder is due to a faulty control of the sinus node.

41. A method of prophylactically or therapeutically treating a mammal for a disturbance of consciousness, which method comprises administering to said mammal a vector comprising and expressing a prophylactically or therapeutically effective amount of an isolated or purified nucleic acid of claim 1, whereupon said mammal is treated for said disturbance of consciousness.

42. The method of claim 41, wherein said disturbance of consciousness is due to a malfunction in thalamic neurons.

43. A method of prophylactically or therapeutically treating a mammal for a disturbance of consciousness, which method comprises administering to said mammal a prophylactically or therapeutically effective amount of a polypeptide of claim 19, whereupon said mammal is treated for said disturbance of consciousness.

44. The method of claim 43, wherein said disturbance of consciousness is due to a malfunction in thalamic neurons.

45. A method of prophylactically or therapeutically treating a mammal for a pain state, which method comprises administering to said mammal a vector comprising and expressing a prophylactically or therapeutically effective amount of an isolated or purified nucleic acid of claim 1, whereupon said mammal is treated for said pain state.

46. A method of prophylactically or therapeutically treating a mammal for a pain state, which method comprises administering to said mammal a prophylactically or therapeutically effective amount of a polypeptide of claim 19, whereupon said mammal is treated for said pain state.

Abstract

Sequences of an I_h ion channel and use thereof

The present invention relates to a nucleic acid, preferably a DNA, comprising at least a part of the sequence of an I_h ion channel. Said sequence may e.g. be derived from a human DNA, a rat DNA, a bovine DNA, a *Drosophila melanogaster* DNA or a sea urchin DNA. Furthermore, the present invention relates to an mRNA molecule which contains the corresponding sequences. The invention further relates to a polypeptide or protein encoded by the nucleic acid.

Furthermore, the invention relates to the use of the inventive nucleic acid and proteins for a screening and/or diagnosing method and to the kits required therefor.

Lastly, the invention relates to the use of one or more nucleic acids and proteins for the treatment and/or prophylaxis of cardiovascular disorders and disturbances of consciousness.

Fig. 1A

Sequence ID No. 4

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CGGGAGAATAGTGCACCAAGGGATGCCCGTGAAATATTAATTAAACGTTTTTAAGAACA  -101
TCATCAAACCCGGGCCCCATCATGAAGGAATAACAAGGCCTTCGAAAAGTATGGGAAACT  -41
GGTCGGCAGGACATCAGCATTATTAATTCTAGGAAACTCATTATGGATAACAAGGAAACT  18
                                            M  D  N  K  E  T  6

AACGGAGAGCTAGAGCAGTCTGATGAGGCCGATCCGTCCGGTCAAAACCTTGATGATGGG  78
 N  G  E  L  E  Q  S  D  E  A  D  P  S  G  Q  N  L  D  D  G  26

GAAACCGATAGCAAACAAGAAGAGAATCTCATCAACGTTAGCCCGCCAAAAACACCGCCA  138
 E  T  D  S  K  Q  E  E  N  L  I  N  V  S  P  P  K  T  P  P  46

GGTCCTCCTCCTCCTCTAAAGAATGGAGGAAGGGGTCAGAAACCGCCCAAAATCCCAATA  198
 G  P  P  P  P  L  K  N  G  G  R  G  Q  K  P  P  K  I  P  I  66

TGTCATCAAAATGGAAAGCTCCCCAAGGAAGTTGAATGGACAGAAGACAGAGGCGAAGAC  258
 C  H  Q  N  G  K  L  P  K  E  V  E  W  T  E  D  R  G  E  D  86

AGAAAGGATAGTCTCACTCTTCAATCAAAGCTAGATCACGGGGCATACACGGATGAGAAA  318
 R  K  D  S  L  T  L  Q  S  K  L  D  H  G  A  Y  T  D  E  K  106
          ▲
CAGGATCTTCTAACATATCTTGACCGTCACGGCATCAACAGTCCAGTCAAGCTAACACCA  378
 Q  D  L  L  T  Y  L  D  R  H  G  I  N  S  P  V  K  L  T  P  126

GATGAAACTGGAGGGAGCAGTGCTTTGGATATTCTTGGATTATTGAAGAGAGGGACACT  438
 D  E  T  G  G  S  S  A  L  D  I  L  G  I  I  E  E  R  D  T  146

GGTGCACTAGGCTCTGATCCCTCATCCACTATGCAGGCCATGGCTAAACCTGTAGGCTTT  498
 G  A  L  G  S  D  P  S  S  T  M  Q  A  M  A  K  P  V  G  F  166

CTGCAGAGGCAGCTATGGACTGTCCTCCAACCTTCAGACAATAGACTCTCCATGAAACTT  558
 L  Q  R  Q  L  W  T  V  L  Q  P  S  D  N  R  L  S  M  K  L  186
                                                   •
TTCGGAAGCAAGAAAGGGTTACAAAAGGAAAAATATCGGCTGAGGAAGGCGGGGGTTCTT  618
 F  G  S  K  K  G  L  Q  K  E  K  Y  R  L  R  K  A  G  V  L  206
       •
                         ---------------------- S1 ------
ATCATTCATCCATGTAGTCATTTCAGATTTTACTGGATCTACTGATGCTGTGCCTGATC  678
 I  I  H  P  C  S  H  F  R  F  Y  W  D  L  L  M  L  C  L  I  226

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ATGGCAAACGTCATCCTCCTACCCGTCGTCATTACTTTCTTCCACAACAAGGACATGAGT  738
 M  A  N  V  I  L  L  P  V  V  I  T  F  F  H  N  K  D  M  S  246

      --------------------------------- S2 ------
ACGGGTTGGCTCATCTTTAATTGCTTCTCAGATACCTTCTTCATTCTCGATCTCATCTGC  798
 T  G  W  L  I  F  N  C  F  S  D  T  F  F  I  L  D  L  I  C  266

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AACTTTCGGACCGGCATCATGAATCCGAAGTCGGCCGAACAGGTGATCCTCAACCCCCGT  858
 N  F  R  T  G  I  M  N  P  K  S  A  E  Q  V  I  L  N  P  R  286

                            ----------------- S3 ------
CAAATCGCCTATCATTATCTCCGTTCATGGTTCATCATCGATCTCGTGTCTTCCATCCCC  918
 Q  I  A  Y  H  Y  L  R  S  W  F  I  I  D  L  V  S  S  I  P  306
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ATGGACTACATCTTCCTCCTCGCTGGCGGCCAGAACCGTCACTTCCTCGAGGTGTCCCGA   978
 M  D  Y  I  F  L  L  A  G  G  Q  N  R  H  F  L  E  V  S  R    326

                                S4
GCCCTCAAGATACTGCGCTTTGCCAAGCTCCTCAGTCTTCTTCGACTCCTGCGTCTGTCC   1038
 A  L  K  I  L  R  F  A  K  L  L  S  L  L  R  L  L  R  L  S    346

AGGCTCATGCGGTTCGTCAGTCAATGGGAACAGGCCTTCAACGTAGCCAATGCCGTCATC   1098
 R  L  M  R  F  V  S  Q  W  E  Q  A  F  N  V  A  N  A  V  I    366

                                S5
CGGATCTGTAATCTAGTGTGTATGATGCTTCTGATTGGCCATTGGAATGGCTGCCTTCAA   1158
 R  I  C  N  L  V  C  M  M  L  L  I  G  H  W  N  G  C  L  Q    386

TATCTCGTGCCCATGCTGCAAGAATACCCCGACCAATCATGGGTCGCCATTAATGGCCTT   1218
 Y  L  V  P  M  L  Q  E  Y  P  D  Q  S  W  V  A  I  N  G  L    406

                                                  Pore
GAGCACGCTCATTGGTGGGAGCAGTATACATGGGCACTCTTCAAAGCCCTTTCGCACATG   1278
 E  H  A  H  W  W  E  Q  Y  T  W  A  L  F  K  A  L  S  H  M    426

CTCTGTATCGGGTACGGCAAGTTCCCCCCTCAAAGCATCACCGATGTCTGGCTAACGATT   1338
 L  C  I  G  Y  G  K  F  P  P  Q  S  I  T  D  V  W  L  T  I    446

                              S6
GTCAGTATGGTGTCCGGTGCGACCTGCTTCGCCCTGTTCATCGGACACGCTACCAATCTC   1398
 V  S  M  V  S  G  A  T  C  F  A  L  F  I  G  H  A  T  N  L    466

ATCCAGTCCATGGACTCCTCCAGCAGGCAATACCGTGAGAAGTTGAAACAAGTTGAAGAG   1458
 I  Q  S  M  D  S  S  S  R  Q  Y  R  E  K  L  K  Q  V  E  E    486

TACATGCAGTATCGCAAGCTACCGTCCCACCTACGAAACAAGATCCTCGATTACTACGAG   1518
 Y  M  Q  Y  R  K  L  P  S  H  L  R  N  K  I  L  D  Y  Y  E    506

TACCGATACCGAGGAAAGATGTTTGATGAGAGGCATATCTTTCGAGAAGTGTCGGAGAGT   1578
 Y  R  Y  R  G  K  M  F  D  E  R  H  I  F  R  E  V  S  E  S    526

ATACGACAGGATGTCGCAAACTACAATTGTCGCGACCTGGTCGCATCCGTCCCTTTCTTC   1638
 I  R  Q  D  V  A  N  Y  N  C  R  D  L  V  A  S  V  P  F  F    546

GTCGGTGCCGACTCAAACTTCGTCACCCGTGTGGTGACGCTGCTCGAATTCGAGGTCTTC   1698
 V  G  A  D  S  N  F  V  T  R  V  V  T  L  L  E  F  E  V  F    566

CAACCCGCTGACTATGTTATACAGGAAGGTACTTTCGGTGATCGCATGTTCTTCATCCAG   1758
 Q  P  A  D  Y  V  I  Q  E  G  T  F  G  D  R  M  F  F  I  Q    586

CAGGGCATCGTCGACATCATCATGTCCGACGGCGTCATCGCCACGTCACTCAGTGACGGC   1818
 Q  G  I  V  D  I  I  M  S  D  G  V  I  A  T  S  L  S  D  G    606

                         cNMP binding site
TCATATTTTGGCGAAATCTGCCTGCTTACCCGTGAGCGCCGCGTGGCATCGGTGAAGTGC   1878
 S  Y  F  G  E  I  C  L  L  T  R  E  R  R  V  A  S  V  K  C    626

GAGACCTACTGCACGCTCTTCTCGCTCTCCGTCCAGCATTTCAACCAAGTGCTCGACGAG   1938
 E  T  Y  C  T  L  F  S  L  S  V  Q  H  F  N  Q  V  L  D  E    646
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Fig. 1B, 1C

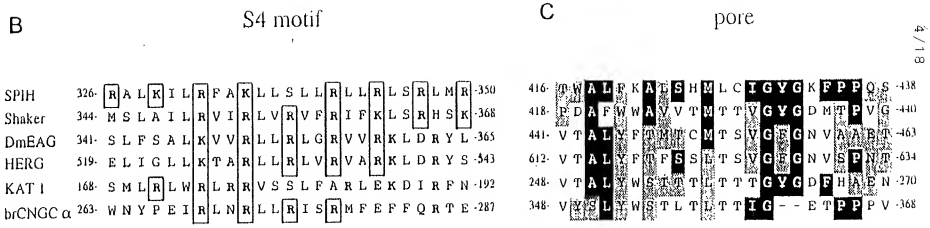

Fig. 1D

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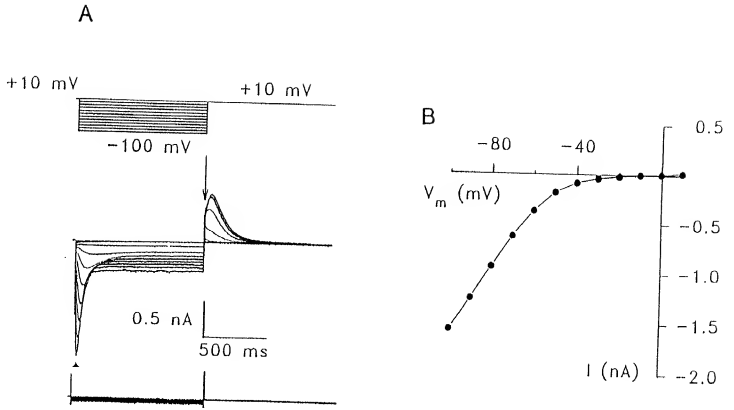
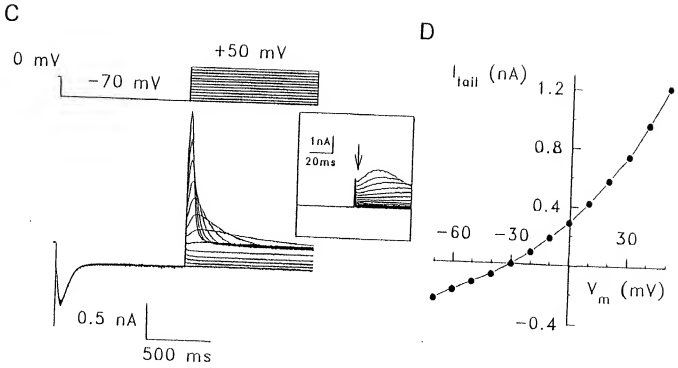
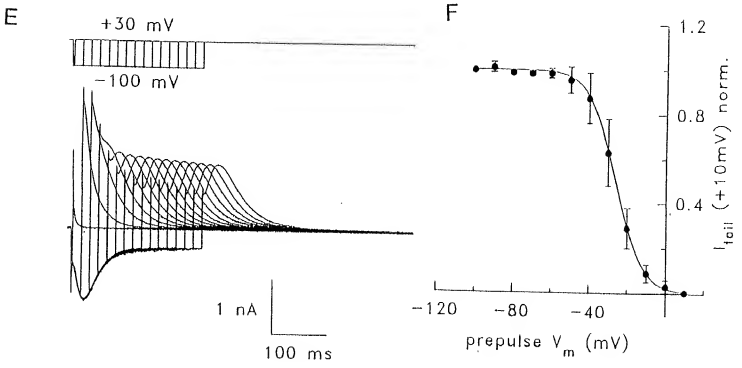
Fig. 2A, 2B



Fig, 2C, 2D



Fig, 2E, 2F



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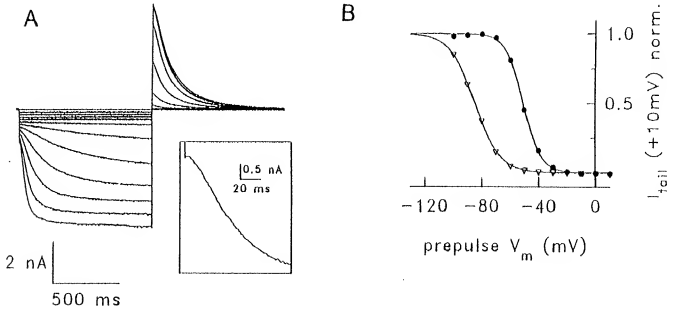

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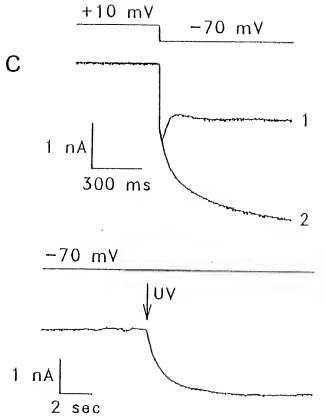

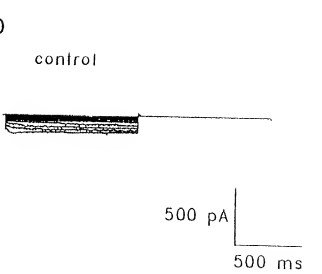

Fig. 3E, 3F

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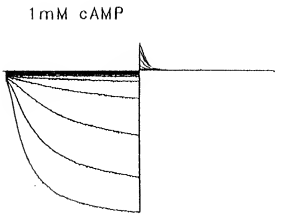

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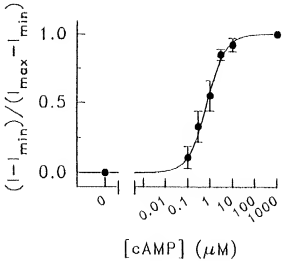

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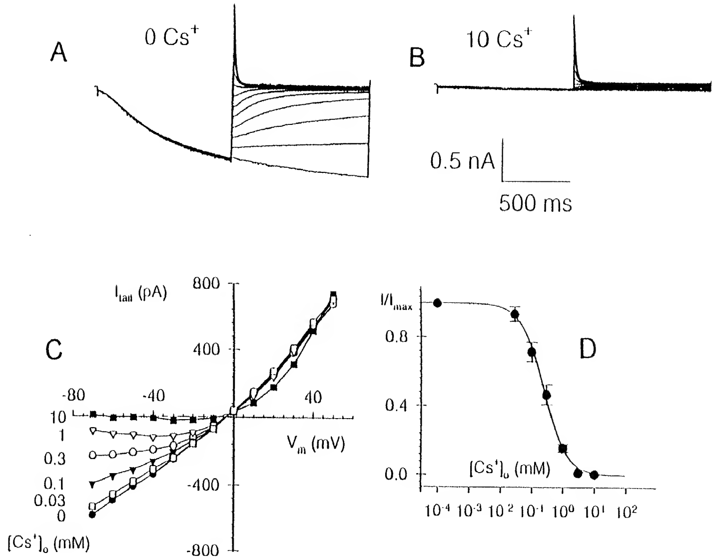
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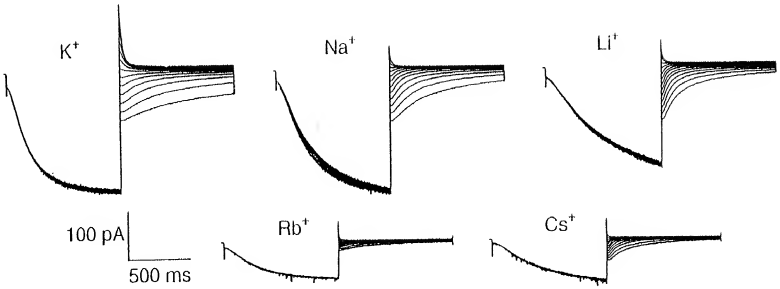

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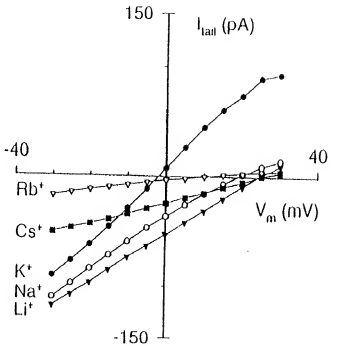

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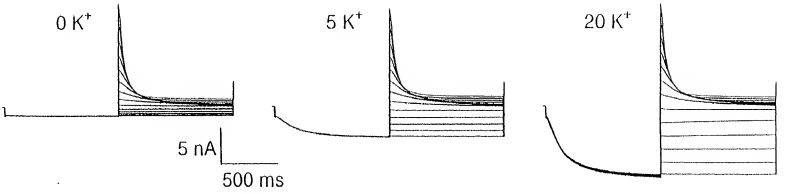

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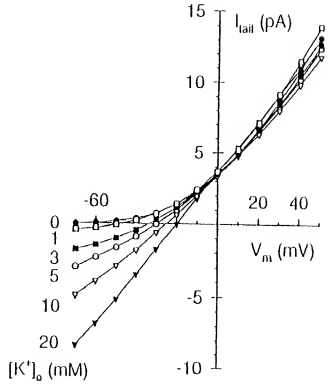

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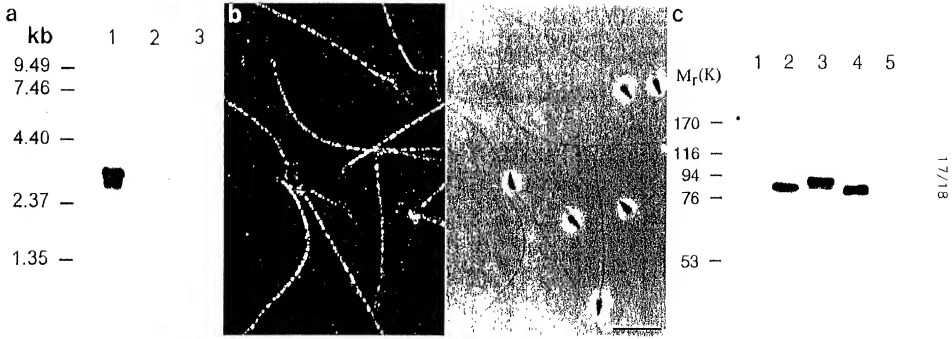

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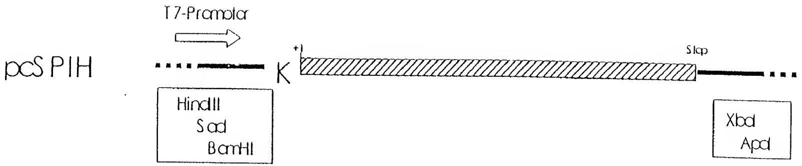

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Pro Ile Cys His Gln Asn Gly Lys Leu Pro Lys Glu Val Glu Trp Thr
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Ala Lys Pro Val Gly Phe Leu Gln Arg Gln Leu Trp Thr Val Leu Gln
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Trp Ala Leu Phe Lys Ala Leu Ser His Met Leu Cys Ile Gly Tyr Gly
            420                 425                 430

Lys Phe Pro Pro Gln Ser Ile Thr Asp Val Trp Leu Thr Ile Val Ser
        435                 440                 445

Met Val Ser Gly Ala Thr Cys Phe Ala Leu Phe Ile Gly His Ala Thr
    450                 455                 460

Asn Leu Ile Gln Ser Met Asp Ser Ser Ser Arg Gln Tyr Arg Glu Lys
465                 470                 475                 480

Leu Lys Gln Val Glu Glu Tyr Met Gln Tyr Arg Lys Leu Pro Ser His
                485                 490                 495

Leu Arg Asn Lys Ile Leu Asp Tyr Tyr Glu Tyr Arg Tyr Arg Gly Lys
            500                 505                 510

Met Phe Asp Glu Arg His Ile Phe Arg Glu Val Ser Glu Ser Ile Arg
        515                 520                 525

Gln Asp Val Ala Asn Tyr Asn Cys Arg Asp Leu Val Ala Ser Val Pro
    530                 535                 540

Phe Phe Val Gly Ala Asp Ser Asn Phe Val Thr Arg Val Val Thr Leu
545                 550                 555                 560

Leu Glu Phe Glu Val Phe Gln Pro Ala Asp Tyr Val Ile Gln Glu Gly
                565                 570                 575

Thr Phe Gly Asp Arg Met Phe Phe Ile Gln Gln Gly Ile Val Asp Ile
            580                 585                 590

Ile Met Ser Asp Gly Val Ile Ala Thr Ser Leu Ser Asp Gly Ser Tyr
        595                 600                 605

Phe Gly Glu Ile Cys Leu Leu Thr Arg Glu Arg Arg Val Ala Ser Val
    610                 615                 620

Lys Cys Glu Thr Tyr Cys Thr Leu Phe Ser Leu Ser Val Gln His Phe
625                 630                 635                 640

Asn Gln Val Leu Asp Glu Phe Pro Ala Met Arg Lys Thr Met Glu Glu
                645                 650                 655

Ile Ala Val Arg Arg Leu Thr Arg Ile Gly Lys Glu Ser Ser Lys Leu
            660                 665                 670

Lys Ser Arg Leu Glu Ser Pro Thr Ile Arg Asp Thr Ala Pro Leu Phe
        675                 680                 685

Pro Ile Pro Pro Asp Thr Pro Ser Phe Val Thr Asp Ile Glu Lys Asn
    690                 695                 700

Arg Phe Phe Gly Asp Asp Thr Asp Asp Val His Ile Arg Thr Arg Val
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705                 710                 715                 720

Asp Val Glu Arg Gly Ser His Glu Asn Val Ile Ala Ile Met Asp Gly
                725                 730                 735

Ser Leu Ser Asp Leu Arg Met Glu Asn Glu Ile Gln Ala Arg Lys Ser
            740                 745                 750

Ser Ser Gly Lys Arg Arg Lys Phe Gln Gln Gln Thr Thr Glu Leu
        755                 760                 765
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